Reactive oxygen species-scavenging system is involved in L-amino acid oxidase accumulation in *Pseudoalteromonas* sp. B3

Zhiliang Yu¹ · Yanyan Yang¹ · Yangsheng Wang¹ · Jianhua Yin¹ · Juanping Qiu¹

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Abstract To date, the mechanisms underlying the flavo-protein L-amino acid oxidase (LAAO) accumulation in cells remain unclear. In this study, using LAAO-producer *Pseudoalteromonas* spp. as model organisms, we found that the cell biomass is negatively associated with LAAO accumulation, whereas the LAAO accumulation is positively associated with the reactive oxygen species (ROS)-scavenging capability. The expression levels of ROS-scavenging-associated genes were up-regulated with LAAO accumulation in *Pseudoalteromonas* cells, which is presumably due to the requirement for the removal of LAAO-induced ROS. Exogenous H₂O₂ exposure experiment supported that the ROS-scavenging system is associated with LAAO accumulation in *Pseudoalteromonas*. All these observations indicate that ROS-scavenging capacity determines LAAO accumulation in bacterial cells. Our results shed a light on understanding the mechanism underlying controlling and adapting to LAAO accumulation in *Pseudoalteromonas*. Besides, our findings are critical to the improvement of heterologous expression of active LAAO in the future.

Keywords LAAO · ROS-scavenging system · *Pseudoalteromonas* · Oxidation adaption

Introduction

L-Amino acid oxidase (LAAO; EC 1.4.3.2) can stereospecifically convert L-amino acid to the corresponding α -keto acid with release of ammonia and hydrogen peroxide (H₂O₂) (Nakano and Danowski 1966). LAAO exists in different organisms (Chacón-Verdú et al. 2013; Gómez et al. 2008) with broad biological functions, such as antimicrobial (Gómez et al. 2008; James et al. 1996; Kitani et al. 2007), anti-HIV (Zhang et al. 2003), antitumor cell (Treshalina et al. 2000), inducing cell apoptosis (Suhr and Kim 1996), etc. Increasing studies show that biological roles of LAAO are associated with H₂O₂ generated by itself (Lucas et al. 2006; Yu and Qiao 2012). As one kind of reactive oxygen species (ROS), H₂O₂ is usually harmful to LAAO-producers themselves. Hence, LAAO is defined as toxic protein. Although LAAO showed broad applications in biosensor, biotransformation, and bleaching (Yu and Qiao 2012), its large-scale application is currently limited due to the challenge in heterologous expression derived from its toxicity to host cells (Yu et al. 2014a). Hence, how the LAAO-producing organisms control and adapt to the LAAO accumulation is important, but not very clear yet.

It has been reported that bacteria can protect themselves from H₂O₂ with scavenging enzymes and repair system (Mishra and Imlay 2012). When bacterial cell is exposed to low dose of exogenous H₂O₂, catalase in the cells will be induced and H₂O₂ can be effectively removed (Christman et al. 1985). It has been further found that a *Pseudomonas aeruginosa* mutant with the altered catalase-encoding gene is extremely sensitive to exogenous H₂O₂ (Hassett et al. 1999). These findings demonstrate that catalase is extraordinarily important for the protection of cells from H₂O₂ (Imlay 2013). Alkyl hydroperoxide reductase (Ahp)

Zhiliang Yu and Juanping Qiu contributed equally to this work.

✉ Zhiliang Yu
zlyu@zjut.edu.cn

✉ Juanping Qiu
qiujiaping@zjut.edu.cn

¹ College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, China

is another important enzyme for defense of peroxides. It was found that Ahp and RpoS (general stress resistance σ factor) in *P. aeruginosa* involve the defense of oxidative stress (Ochsner et al. 2000; Suh et al. 1999). Taken together, it is interesting to know whether and how LAAO accumulation is associated with ROS-scavenging capacity.

LAAO has been found in marine *Pseudoalteromonas* spp. (Chen et al. 2010; Isnansety and Kamei 2003; James et al. 1996) with important roles in cell dispersal and colonization (Lourenco et al. 2008), suggesting its important ecological function in marine niches. Recently, a marine bacterium *Pseudoalteromonas* sp. B3 was isolated and found to produce LAAO (Yu et al. 2013). Interestingly, a spontaneous mutant of strain B3, designated as R3, was found to have higher LAAO activity (Yu et al. 2013). In this study, we, for the first time, demonstrated that the differentiated LAAO accumulation between strain B3 and strain R3 is due to the differentiated ability in ROS removal, thus revealing that the ROS-scavenging capacity determines LAAO accumulation in *Pseudoalteromonas* cells. Our findings would help in uncovering the mechanisms underlying controlling and adapting to LAAO biosynthesis in *Pseudoalteromonas*, as well as for improving its heterologous expression in the future.

Materials and methods

Bacterial strains and growth conditions

LAAO-producing bacterium *Pseudoalteromonas* sp. B3 was isolated from marine sludge sample (Yu et al. 2013). *Pseudoalteromonas* sp. R3 is a spontaneous mutant of strain B3 with higher LAAO activity, which has been determined by detecting their generated α -keto acid and H_2O_2 (Yu et al. 2013). Strain B3 and strain R3 share completely identical sequences of 16S rRNA gene and *lao* (encoding LAAO), which have been confirmed by sequencing. Both strains were cultured on solid marine medium (MM) (3 g/L of yeast extract, 5 g/L of tryptone, 30 g/L of sea salt, and 20 g/L of agar) at 28 °C overnight. Next, a ring of cell was inoculated to 5 mL of broth MM (solid medium without agar) in a test tube for incubation at 28 °C for 12 h with a shaking at 150 rpm. Finally, 1 mL of cell culture was transferred to 50 mL of broth MM in 250 mL flask and the mixture was incubated at 28 °C for required time with a shaking at 150 rpm for LAAO accumulation.

Prussian blue agar assay for determination of LAAO activity

Prussian blue agar assay was used to determine LAAO activity by detecting the formed H_2O_2 (Yu et al. 2013, 2014b). In brief, 2 mL of the cell cultures was mixed with 2 mL of 10 mM L-Leu for oxidation at 30 °C. After 1 h incubation, 50 μ L of supernatant was added to the punched circular well which is 6 mm in diameter on Prussian blue agar [1.0 g/L $FeCl_3 \cdot 6H_2O$, 1.0 g/L potassium hexacyanoferrate (III), 2% agar, pH 7.5]. LAAO activity was determined by extracting the correlation between the diameter of the formed Prussian blue halo and its corresponding H_2O_2 concentration. One unit (U) of LAAO activity is defined as the amount of enzyme that catalyzes the formation of 1 mM H_2O_2 /h at 30 °C.

Measurement of bacterial biomass

Unless otherwise stated, the bacterial biomass of cell treated with or without H_2O_2 was determined based on the value of either OD_{600nm} or colony forming unit (CFU). After washing and appropriate dilution with fresh broth MM, the OD_{600nm} value of sample was detected by UV–Vis spectrophotometer (Lengguang Tech, China). Otherwise, 200 μ L of the appropriately diluted samples was poured into the double-layer MM agar and cultured at 30 °C for 24 h. Then, the CFU on the agar plate was analyzed.

Detection of intracellular ROS

The probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) can be intracellularly deacetylated by a non-specific esterase and further oxidized by ROS to fluorescent compound inside (Li et al. 2006). In this study, the intracellular level of ROS was detected based on the oxidation of DCFH-DA using a ROS assay kit (Beyotime, Jiangsu, China), according to manufacturer's instruction. In general, 1 mL of bacterial cell (10^7 CFU/mL) was treated with or without 1.5 mM H_2O_2 for 30 min at 30 °C. After washing twice with fresh broth MM, the cells were resuspended in 1 mL of 10 μ M DCFH-DA for 20 min incubation at 37 °C. Next, the cells were collected after centrifugation at 4000g for 5 min and washed twice with fresh broth MM. After resuspension in 1 mL of fresh broth MM, 200 μ L of the cells was detected on a multimode reader (Spectramax M5, Molecular Devices, USA) with excitation and emission of 488 and 525 nm, respectively.

Determination of lipid peroxidation

Lipid peroxidation was evaluated based on the formation of malondialdehyde (MDA), which was detected using thio-barbituric acid assay (Ohkawa et al. 1979). In brief, 1 mL of bacterial cell (10^7 CFU/mL) was treated with or without 1.5 mM H_2O_2 for 30 min at 30 °C. After washing twice with fresh broth MM, the cells were resuspended in 100 μ L of breaking solution containing 5% SDS and 250 mM EDTA for incubation at 37 °C for 30 min. Next, according to manufacturer's instruction, MDA concentration was determined using MDA assay kit (Beyotime, Jiangsu, China) on a multimode reader (Spectramax M5, Molecular Devices, USA) at a wavelength of 532 nm.

Cellular tolerance to H_2O_2

Cells at OD_{600nm} of 0.6 were subjected to H_2O_2 with different concentrations for 30 min at 30 °C on a shaker. Then, cells were collected after centrifugation at 4000g for 5 min, washed twice with fresh broth MM, and resuspended with fresh broth MM. Next, the survival rate of cells was evaluated by counting CFU on solid MM plate after incubation at 30 °C for 24 h.

Measurement of relative expression level of gene using quantitative real-time PCR (qRT-PCR)

The qRT-PCR was applied to quantification of the relative expression level of *lao* and ROS-scavenging-associated genes (Pfaffl 2001; Yu et al. 2015a). Unless otherwise specified, the cultured *Pseudoalteromonas* cells were collected after centrifugation at 8000g for 10 min and the total RNA was prepared using RNAiso Plus kit (TaKaRa, Dalian, China). RNA integrity was detected according to the ratio of OD_{260nm}/OD_{280nm} , and 500 ng of RNA sample was applied to cDNA amplification in 10 μ L using PrimeScriptTM RT Master Mix kit (TaKaRa, Dalian, China).

After several dilutions, the transcribed cDNA was used as a template for the amplification of target gene fragment using SYBR green *Premix Ex Taq*TM kit (TaKaRa, Dalian, China). All the primers (Table 1) were designed according to the genome sequence of *Pseudoalteromonas* sp. R3 (GenBank access number LJDF000000000) (Yu et al. 2015b). The PCR was run on CFX Connect Real-Time System (Bio-Rad, CA, USA) using amplification system consisting of an initial 10 min denaturation at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C and 30 s annealing/elongation at 60 °C. Immediately after final cycle, melting curve was analyzed based on the observation of melting temperature and the reaction specificity was accordingly determined.

The cycle threshold (C_T) per sample was determined using STATVIEW software, which automatically set the threshold signal at log phase of the amplification curve. Different dilutions for each sample were applied for obtaining a linear regression between C_T value and log of the cDNA. The amplification efficiency of gene was resulted from the linear regression slope. As an internal control, 16S rRNA gene fragment with 108 bp was amplified using forward primer (5'-AAGAAG-CACCGGCTAACTCC-3') and reverse primer (5'-TCTCGCTTAATCAACCGCCT-3'). The relative expression level of target gene over that of 16S rRNA gene was determined using the comparative C_T method (Schmittgen and Livak 2008).

Data analysis

Unless otherwise stated, triplicate reactions per experiment were performed. All the data were presented as mean $\pm$ standard error and statistical significance was analyzed based on the analysis of variance (ANOVA) followed by the Dunnett's post hoc test using StatView 5.0 software. When the probabilities were less than 0.05, 0.01, and 0.001, the differences were considered signifi-

Table 1 Sequences of the pair primers for real-time PCR

Genes	Nucleotide sequences (5'-3')		Product sizes (bp)
	Forward primer	Reverse primer	
<i>lao</i> *	ATACGCCAAGTGCCTCAGTG	TCCGTCAGCCCGTTAAAGTC	114
<i>rpoS</i>	CTCGCACCATTCGCTTACCT	TGACTCTGCGATTTCCTCCG	119
<i>ahp</i>	GGCGTTGAAGTGATTCGGTC	TTACCTGTCGGGTACCGAT	116
<i>cat</i>	CTGTGCAGGCACGCTTTAAC	TAACACCGGCATTCTTGCT	117

*GenBank Access Number: *lao* (WP_054014055.1); *rpoS* (WP_054016062.1); *ahp* (WP_046006053.1); *cat* (WP_054013490.1)

cant, very significant, and extremely significant, respectively.

Results

Differentiated LAAO activity between strain B3 and strain R3

As shown in Fig. 1, strain B3 and strain R3 had similar growth pattern. When incubated in MM broth, cells rapidly increased from 4 to 12 h and decreased from 12 to 24 h, followed by a further decrease in the remaining period. In comparison, strain R3 grew much faster and had higher cell biomass.

Consistent with our previous study (Yu et al. 2013), strain B3 and strain R3 had different patterns in LAAO accumulation. LAAO activity in strain B3 was detectable only after incubation for 12 h, and remained steady at around 0.4 U later on. However, LAAO activity in strain R3 was detectable after incubation for 4 h, increased dramatically from 16 to 32 h with maximum of 1.84 U, and then decreased. Strain R3 showed a steady increase in LAAO activity per cell biomass in the incubation period ranging from 12 to 40 h, much faster and higher than that of strain B3. These data indicate that strain B3 and strain R3 have the differentiated LAAO accumulation and the latter yields relatively high LAAO activity.

Strain R3 had lower intracellular ROS than strain B3

LAAO can oxidize L-amino acid to generate H_2O_2 , which is an important class of ROS (Nakano and Danowski 1966; Storz and Imlay 1999). Therefore, the levels of intracellular ROS in strain B3 and strain R3 were determined by detecting the fluorescence signal of 2,

Fig. 1 Differentiated OD_{600nm} and LAAO activity between strain B3 and strain R3 during incubation

Fig. 2 Changes in the intracellular ROS level between strain B3 and strain R3 during incubation

7-dichlorofluorescein diacetate (DCF-DA). As shown in Fig. 2, the intracellular ROS in both strains drastically increased first and then rapidly decreased. The maximum level in strain B3 and strain R3 appeared after incubation for 16 and 12 h, respectively. Notably, strain R3 had higher LAAO activity per cell biomass since 16 h, but much lower ROS accumulation per cell biomass than strain B3. These results suggest that strain R3 has stronger capacity to scavenge LAAO-induced ROS than strain B3, although the former yields relatively high level of LAAO activity.

Strain R3 had higher transcriptional levels of *lao* and ROS-scavenging genes than strain B3

The relative expression levels of *lao* and ROS-scavenging genes (*rpoS*, *ahp*, and *cat*) were determined by qRT-PCR (Pfaffl 2001; Yu et al. 2015a). Figure 3 shows that the transcriptional level of *lao* in both strains first increased and then reached maximum at 32 h. These results were in agreement with the increased LAAO activities in both strains (Fig. 1). Besides, the transcriptional level of *lao* in strain R3 was much higher than that in strain B3 within the almost entire incubation period, probably explaining that LAAO activity in strain R3 was much higher than that in strain B3.

Similarly, the expression levels of all the important ROS-scavenging genes gradually increased with the increase of LAAO activity in both strains. Most probably, LAAO accumulation leads to the production of H_2O_2 and then induces the expression of ROS-scavenging genes for response. Interestingly, the expression levels of all ROS-scavenging genes in strain R3 were higher than those in strain B3. Further study indicated that the addition of 1.5 mM H_2O_2 had no effect on the *lao* expression, but up-regulated the expression of ROS-scavenging genes in both strains (Fig. 4). The expression levels of all ROS-scavenging genes in strain R3 were higher than those in strain

Fig. 3 Differentiated relative gene expression level with LAAO accumulation between strain B3 and strain R3 during incubation. **a** *lao*, **b** *rpoS*, **c** *ahp*, **d** *cat*

Fig. 4 Relative expression level of *lao* and ROS-scavenging genes in strain B3 (**a**) and strain R3 (**b**), when exposed to 1.5 mM of exogenous H_2O_2

B3. All these data demonstrate that ROS-scavenging system in strain R3 is much more sensitive to H_2O_2 than that in strain B3. Accordingly, strain R3 has relatively stronger ability to remove LAAO-induced H_2O_2 for survival and thus presumably has relatively high LAAO accumulation.

Strain R3 could tolerate higher level of H_2O_2 than strain B3

To analyze the effect of exogenous H_2O_2 on the cell survival, CFUs were calculated. The survival rate of both strains decreased with the increase of H_2O_2 concentration

Fig. 5 Differentiated alteration in strain B3 and strain R3, when exposed to exogenous H_2O_2 . **a** Survival rate at different concentrations of H_2O_2 ; **b** accumulation of intracellular ROS at 1.5 mM of H_2O_2 ; **c** lipid peroxidation according to MDA level at 1.5 mM of H_2O_2

(Fig. 5a), implying that H_2O_2 can induce the cell death of *Pseudoalteromonas* in a concentration-dependent manner. Both strains had the similar ability to tolerate H_2O_2 with concentrations below 0.3 mM. However, the survival rate of strain B3 and strain R3 was distinct when exposed to H_2O_2 with concentrations above 0.3 mM. Strain R3 showed higher survival rate than strain B3 with the

increase of H_2O_2 concentration, indicating that strain R3 has relatively stronger ability to tolerate H_2O_2 with high concentration.

The intracellular ROS accumulation induced by H_2O_2 was determined. When exposed to 1.5 mM H_2O_2 , the fluorescence signal increased from 1249 to 2151 in strain B3 (72.2% increased) and from 1025 to 1330 in strain R3 (29.8% increased) (Fig. 5b). The enhanced fluorescence signal in strain R3 was less than that in strain B3, indicating that strain R3 accumulates less amount of H_2O_2 -induced ROS than strain B3. To confirm this, ROS-mediated lipid peroxidation was evaluated by detecting MDA level (Ohkawa et al. 1979). Consistently, the MDA level increased in both strains upon exposure to H_2O_2 (Fig. 5c). The MDA increase in strain R3 (from 1.29 to 3.61 μ M, 178% increased) was much less than that in strain B3 (from 1.68 to 6.09 μ M, 262% increased). These data collectively implicate that strain R3 has relatively higher ability to scavenge H_2O_2 -induced ROS.

Discussion

Marine bacterium *Pseudoalteromonas* sp. B3 has the ability to produce LAAO (Yu and Qiao 2012). Strain R3, a spontaneous mutant of strain B3, displayed higher LAAO activity (Fig. 1). Our results in Fig. 1 show that the cell biomass of both strains was negatively correlated with the LAAO accumulation. Most probably, the accumulated LAAO resulted in the production of H_2O_2 (Fig. 2), which is hazardous to *Pseudoalteromonas* cells. The ROS generation is inevitable under aerobic and LAAO-present condition (Boehm et al. 1976). Only some microorganisms can survive under oxidative stress by effectively removing ROS, but the others grow poorly, suffer from mutagenesis, or even die when the balance between ROS formation and removal in cells is disturbed (Mishra and Imlay 2012).

It is not surprising that LAAO accumulation up-regulated the expression levels of ROS-scavenging-associated genes (Fig. 3), indicating that *Pseudoalteromonas* cells need ROS-scavenging system to remove LAAO-induced ROS for survival. Interestingly, strain R3 had higher LAAO accumulation than strain B3 (Fig. 1). Conversely, strain R3 showed less ROS level with LAAO accumulation than strain B3 (Fig. 2), most probably due to the fact that the increase of expression level of ROS-scavenging genes in strain R3 is much higher than that in strain B3 (Fig. 3). Consequently, LAAO-induced ROS in strain R3 can be more effectively removed than that in strain B3. H_2O_2 exposure experiments further confirmed that ROS-scavenging system in strain R3 was much more effectively induced by H_2O_2 than that in strain B3 (Fig. 4). Therefore, H_2O_2 -induced ROS level in strain R3 was relatively low

(Fig. 5b). As a result, the level of lipid peroxidation in strain R3 was lower than that in strain B3 (Fig. 5c) and, consequently, the survival rate in strain R3 was relatively high, when exposed to H_2O_2 (Fig. 5a).

LAAO is toxic for or has negative effect on the growth of host cells. However, studies on unraveling the mechanisms regarding the adaption to LAAO accumulation are unclear hitherto. Our results demonstrate that LAAO accumulation will result in the increase of ROS and consequently lead to damage on *Pseudoalteromonas* cells, which is in agreement with the previous report (Lucas et al. 2006). In addition, our data, for the first time, elucidate that ROS-scavenging capability is involved in LAAO accumulation in *Pseudoalteromonas*. LAAO is attracting increasing attentions due to its broad applications in biosensor, biochemistry, physiology, and medicine. However, its nature of toxicity to host cells usually results in insoluble inclusion bodies, when heterologously expressed (Yu and Qiao 2012). Therefore, its large-scale application is still limited. Our findings indicate that enhancing the ROS-scavenging ability of host cells is potentially a useful solution for improving the expression of bioactive recombinant LAAO. We believe that our findings could help in uncovering the mechanisms underlying LAAO biosynthesis, as well as improving the understanding of biological and ecological significance of *Pseudoalteromonas* by controlling and adapting to LAAO activity in marine environments. Moreover, our findings are important to deal with the challenge in heterologous expression of active LAAO in the future.

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Compliance with ethical standards

Conflict of interest We declare that we have no conflict of interest.

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